

Full Paper

Label-free DNA detection by loop-mediated isothermal amplification coupled with quartz crystal microbalance sensor

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Received: 21 November 2014 / Accepted: 4 December 2014 / Published: 15 December 2014

Abstract: Loop-mediated isothermal amplification (LAMP) and quartz crystal microbalance (QCM) sensors were used for DNA detection. Porcine DNA as DNA template was amplified and QCM sensors were then used to measure the changes in frequency in positive and negative samples. Mathematical models were employed to extract features from the sensor recordings by fitting the frequency-time plots using polynomial functions. The polynomial function coefficients of the plots gave valuable information for sample classification. Radar graphs and principle component analysis (PCA) were also applied to samples using these coefficients. Both the radar graphs and the PCA indicated differences between the sample groups when using coefficients of high-order functions.

Keywords: label-free DNA detection, DNA amplification, quartz crystal microbalance, loop-mediated isothermal amplification, DNA

INTRODUCTION

DNA detection is applied in many scientific fields such as medicine (treatment, prevention, etc.), forensic science (paternity test, personal identity, etc.) [1], DNA identity testing (GMOs, illegal organism, etc.) [2, 3] and agriculture (food, genetic resources, etc.) [4]. Devices for analysing DNA during screening tests have gained increasing attention over the past decades. Attempts have been made to improve the performance of the sensing devices to attain several desirable features such as high sensitivity, portability for on-site utilisation, low cost and simple protocols and

equipment. This study proposes a label-free, inexpensive and simple method for classifying DNA samples using quartz crystal microbalance (QCM) sensors together with the loop-mediated isothermal amplification (LAMP) method.

QCM is commonly applied in various kinds of analysis, for example gas detection [5, 6], chemical sensing [7, 8] and bio-sensing [9, 10]. It has many advantages over other sensors, such as its small size, low sample volume requirement and capacity to be implemented in real-time measurements. It can also be used as a label-free sensor with a reasonably high resolution. The resonant frequency of the QCM decreases when there is an increase in mass or viscosity of the sample. The detection of DNA using QCM can be performed by measuring the mass change with immobilised probes on the QCM surface when the target DNA hybridises [11, 12]. Attempts have been made to increase the sensitivity of the sensor by labelling the DNA with colloidal gold [13, 14] or magnetic beads [15] to amplify the change in mass. However, these methods are complicated and require sophisticated procedures and expertise.

Tsirtos and co-workers [16] reported a relationship between the length of DNA and its viscosity. They found that the longer the DNA is, the higher its viscosity gets. This implies that different lengths and shapes of DNA lead to different viscosities. Since the DNA concentration is extremely low in most specimens, amplification is normally required before measurement. Polymerase chain reaction (PCR) is a common and well-known technique for amplifying DNA. Since DNA products from the PCR have the same length from the beginning to the end of the process, the viscosity of the specimen is directly proportional to the amount of the products. Recently, a number of reports have shown that the LAMP technique is able to detect viral DNA and RNA from different types of samples from both humans and animals [17-19]. LAMP is a unique DNA amplification method according to Notomi and colleagues [20]. An interesting point of this technique is its primer binding pattern. It produces various lengths of DNA and a cauliflower-like structure and involves the use of the strand displacement activity of DNA polymerase enzyme that can work at the same temperature as that used for the primer annealing. Compared with PCR, which uses three temperature steps for denaturing, annealing and extension, this is another unique characteristic of LAMP, which can amplify the target DNA at a single temperature. Recently, Hatano and colleagues [21] used a disposable pocket warmer with a styrofoam box as the heat source in LAMP without consuming electricity. This demonstrates the feasibility of using LAMP in field work. It is estimated that LAMP can amplify DNA by up to 10^9 - 10^{10} times in an hour at a single temperature. Owing to the structure and length of LAMP products described above, it is believed that LAMP can induce more changes in viscosity than other methods. This can be handled by analysing LAMP products with the QCM. In this paper, the use of the QCM sensor in classifying positive and negative samples before and after amplification by LAMP is reported.

MATERIALS AND METHODS

Reagents

The stock buffer for LAMP reactions was 10X ThermoPol reaction buffer from New England BioLabs (USA). The working buffer was prepared by diluting this buffer to 1X with deionised water. Four specific primers for porcine DNA detection were designed by Transfusion Medicine Laboratory, Allied Health Sciences, Chulalongkorn University [22]. The lengths of FIP, BIP, F3 and B3 primers were 38, 36, 18 and 18 bp respectively. The target DNA length was 169 bp. All the primers were synthesised by BioDesign Co. (Thailand). The stock buffer (TBE buffer) for gel electrophoresis was 0.9M Tris-borate -0.02M EDTA (pH 8).

DNA Sample Preparation

Porcine genomic DNA was used as the DNA template. Extraction and separation of the porcine DNA template was performed using Wizard[®] Genomic DNA Purification Kit (Promega, USA) [23]. Pork muscle from the supermarket was extracted for genomic DNA following the mouse tail protocol [23]. Except in DNA precipitation, isopropanol incubation was extended to 12 hr.

Test Samples

Positive and negative samples were investigated. The positive sample contained 10 ng/ μ L of porcine genomic DNA template and was labelled as 'Pos'. The negative sample contained 10 ng/ μ L of *Salmonella* genomic DNA template or deionised water and was labelled as 'Neg'. The sample before application of LAMP was labelled as 'Pre' while that after LAMP application was labelled as 'Post'. Four types of samples under investigation were: (1) positive sample before application of LAMP (Pos-Pre), (2) negative sample before application of LAMP (Neg-Pre), (3) positive sample after application of LAMP (Pos-Post), and (4) negative sample after application of LAMP (Neg-Post).

LAMP

LAMP was carried out in a total volume of 25 μ L. The LAMP reaction solution contained 0.4 μ M FIP, 0.4 μ M BIP, 0.2 μ M F3, 0.2 μ M B3, 0.8M betaine (Aldrich Chemical, Germany), 1.4mM dNTP (Invitrogen, Germany), 8U of *Bst* DNA polymerase (New England Biolabs, USA), 20mM Tris-HCl, 10mM KCl, 10mM (NH₄)₂SO₄, 8mM MgSO₄, 0.1% Triton X-100 and 1 μ L of a test sample. In the DNA amplification process, the LAMP reaction solutions were incubated at 60°C for 60 min. in a water bath and then heated at 80°C for 2 min. to terminate the reaction. The LAMP products were then analysed by gel electrophoresis. All four types of samples mentioned above were further analysed by QCMs.

Gel Electrophoresis

A 1.5% agarose gel was prepared using 1.5 g of agar powder (Patanasin Enterprise Ltd, Thailand) in 100 mL of 0.5X TBE buffer solution. The DNA products from LAMP were loaded and run under a constant voltage of 100 volts for 60 min. The gel was stained with SYBR Gold (Invitrogen, Germany) and observed under a UV transilluminator.

QCM Sensors

QCM discs (12 MHz) with a diameter of 12 mm were purchased from Tai Tien Electronics Co. (Thailand). Each disc was coated with layers of chromium and gold electrodes on both sides. First, 50 nm of chromium layer was deposited on both sides of the QCM discs and then 200 nm of gold layer was deposited on the chromium layer. The diameter of the electrode was 4 mm, giving an area of 0.126 cm². The disc was then mounted onto a holder and the contact was made with a silver paste. The electrode surface was subjected to hydrophobic treatment by dipping in 0.5% octadecyltrichlorosilane (Fluka, Switzerland) in toluene for 10 min. The sensors were rinsed with ethanol and dried with nitrogen gas before use. More than 30 QCMs with temperature coefficients of less than 10 Hz/°C were selected for further study.

Frequency Measurement

To measure the resonant frequency of QCM sensors, a laboratory-made measuring system was constructed. The system was composed of an 8-channel measuring circuit capable of making simultaneous measurements by up to 8 sensors. The frequency measuring circuit was a conventional Colpitts oscillator with high speed CMOS-TTL as described by Somboon et al. [24]. The oscillation frequency of the QCM was counted using the 8-bit CMOS microcontroller PIC16F628A. The data from all sensors were transferred to a personal computer through a communication interface driver (MAX232). The system was installed in a temperature-controlled chamber during the measurements. The temperature of the chamber was maintained at $29\pm 1^\circ\text{C}$.

Data Collection

Figure 1 shows a scheme for classifying the samples using QCM. First, the resonant frequencies (f_0) of all blank QCMs were measured and recorded as the background value. Before amplification, positive and negative samples were taken and loaded onto the QCMs. These samples were named Pos-Pre and Neg-Pre respectively. The changes in frequency due to differences in sample composition before amplification were monitored and recorded.

The remaining positive and negative samples were subjected to LAMP. After the reaction was stopped, each sample was loaded onto the QCM. These positive and negative samples after amplification were called Pos-Post and Neg-Post respectively. The changes in frequency of the QCMs were monitored and compared with the samples before amplification to investigate the effect of the amplification.

All measurements were performed by loading a $20\text{-}\mu\text{L}$ sample onto one side of the QCM surface while the other side was left exposed to the air. The changes in frequency after sample loading were monitored and recorded with time until the oscillation stopped.

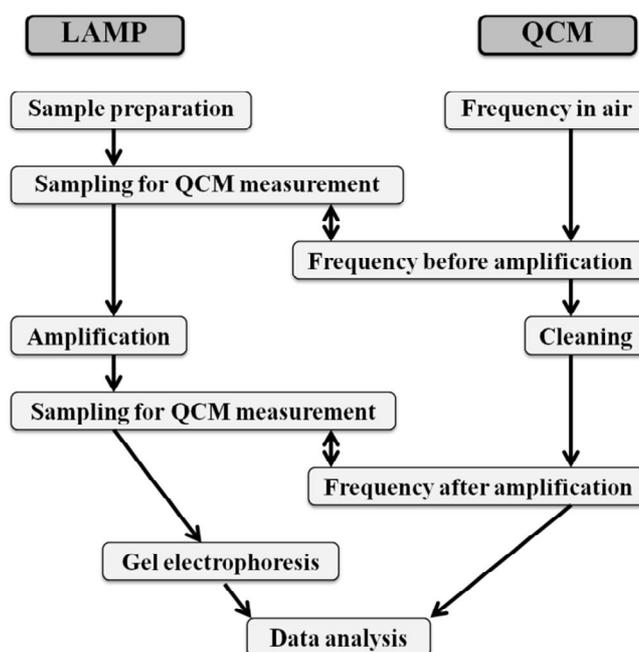


Figure 1. Scheme of QCM sensor for LAMP product detection

Data Analysis

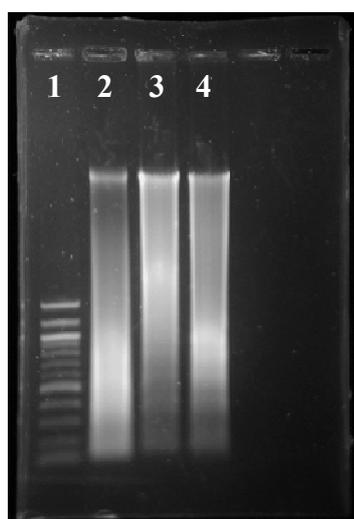
Samples that could not be assessed by gel electrophoresis were excluded from data analysis. Consequently, only 38 samples remained for data analysis as follows: Pos-Pre (n=10), Neg-Pre (n=4), Pos-Post (n=15) and Neg-Post (n= 9).

The four groups of data were classified by comparing the frequency-time plots. Curve fittings were performed on the frequency-time plots using linear, 2nd order polynomial, 3rd order polynomial and 4th order polynomial functions. The coefficients obtained from the 2nd step were then used for the classification by radar plots. Finally, principal component analysis (PCA) was employed to analyse the selected coefficient data.

RESULTS AND DISCUSSION

Validation of LAMP Method

After completing the DNA amplification, the LAMP products were assessed by gel electrophoresis. No bands from the negative samples both before (Neg-Pre) and after (Neg-Post) amplification were detected. Positive samples before amplification (Pos-Pre) also displayed no bands. Although they contained dNTP, primers and DNA template, the amplification did not take place and thus there were no detectable bands. The bands were detected only in the positive samples after LAMP (Pos-Post) as shown in Figure 2. Long-strip bands were observed, showing that various lengths of DNA fragments were produced.



Lane 1 = 100 bp marker

Lane 2 = LAMP product from 10 ng/μL Pork DNA template
(DNA template diluted from 336 ng/μL)

Lane 3 = LAMP product from 10 ng/μL Pork DNA template
(DNA template diluted from 293 ng/μL)

Lane 4 = LAMP product from 10 ng/μL Pork DNA template
(DNA template diluted from 445 ng/μL)

Figure 2. Typical gel electrophoresis pattern of positive samples with DNA template after LAMP (Pos-Post)

Frequency-Time Plots

Figure 3 shows typical frequency-time plots of the four sample groups. The temperature fluctuation during the experiment (2 hr) was less than $\pm 1^\circ\text{C}$; thus, the change in frequency of QCM was not affected by temperature. All four sample groups gave similar responses as follows. At the beginning of the experiment ('a'-'b'), the frequency of QCM was quite stable. During this range of 'a'-'b', the QCM was exposed to air and the frequency recorded at this region was used as the background frequency. A step change in frequency occurred after the sample was loaded (at 'b') onto the sensor surface. This happened within 10 sec. Shortly afterward, the frequency gradually

decreased about 1500 Hz and then an abrupt drop in frequency was observed until the oscillation stopped at point 'c'. The frequency data in the range 'b'-'c' were utilised to characterise Neg-Pre and Pos-Pre samples.

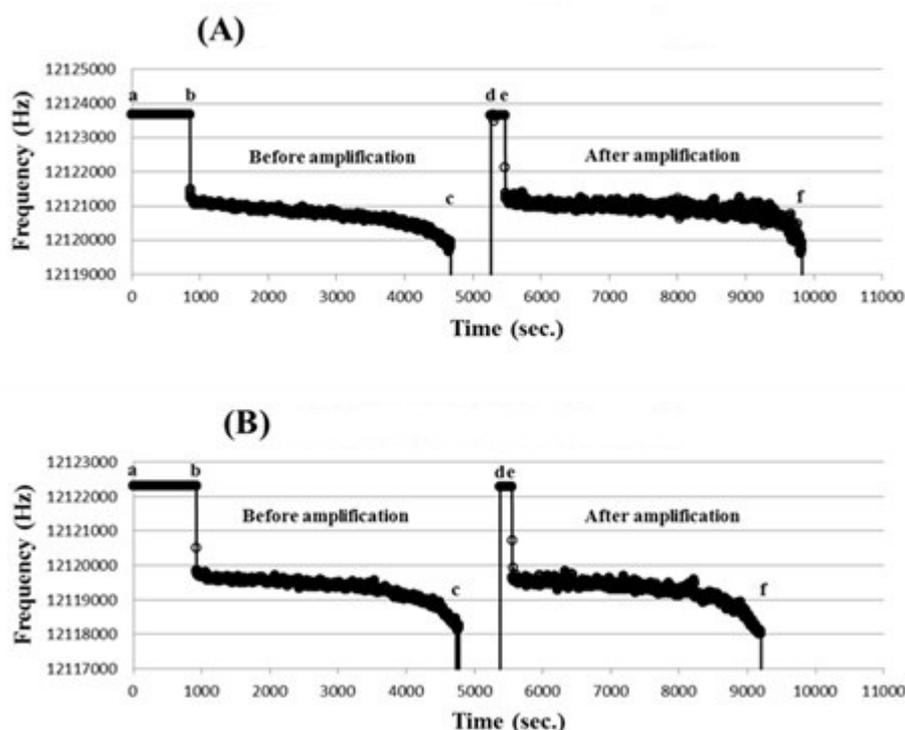


Figure 3. Typical frequency-time plots for samples before and after amplification: (A) negative samples; (B) positive samples

The QCMs were cleaned between each measurement. The four sample groups, after LAMP, were again loaded onto the QCMs. The range 'd'-'e' shows the plot before the loading of the sample subjected to LAMP. In this range, as in range 'a'-'b', the frequency was very stable. The LAMP sample was loaded at point 'e'. A sharp drop of frequency at point 'e' and a gradual decrease in the range 'e'-'f' were observed, similar to that observed at point 'b' and range 'b'-'c'. The data in the range 'e'-'f' were utilised to characterise the Neg-Post and Pos-Post samples. The abrupt change in frequency at the points 'b' and 'e' was the result of sample loading onto the QCM. According to Sauerbrey [25] and Kanazawa and Gordon [26], when the mass, the concentration or the viscosity at the electrode surface increases, the QCM frequency will decrease. The slow change in the ranges 'b'-'c' and 'e'-'f' might be due to a slow precipitation of the sample components on the QCM. Fang and co-workers [27] reported that the DNA appears on the surface of a solution with a low DNA concentration. As the concentration increases, the DNA forms a long chain and penetrates into the solution. After LAMP amplification, a positive sample should have various lengths of DNA fragments. Long-chain DNA fragments are then expected to penetrate into the solution. During evaporation, the long-chain DNA interacts more with the surface electrode than does the pre-LAMP DNA. Indeed, this can be observed from the frequency-time plot (Figure 3), where both the frequency change rate and frequency change value at the point 'f' of the amplified solution (Pos-Post) were higher than those of the negative samples (Neg-Post) and samples before

amplification (Pos-Pre and Neg-Pre). However, it is difficult to distinguish the differences between positive and negative samples before and after amplification using only simple observation.

Data Analysis

Comparison between each group of samples

On detailed examination of Figure 3, it was noticed that the frequency-drop rate of the positive samples (Pos-Pre and Pos-Post) is higher than that of the negative ones (Neg-Pre and Neg-Post). This implies that it is feasible to use this information to distinguish the negative samples from the positive samples. To classify the sample groups, a time-frequency-drop plot was made for each sample group (Figure 4). Since an abrupt change in frequency was observed for 20 sec. after loading the sample at points 'b' and 'e', the data obtained 20 sec. after loading were not used. The data used in the plots were taken after this 20-sec. period. The mean time taken for frequency change of up to 1300 Hz in all the sample groups were used.

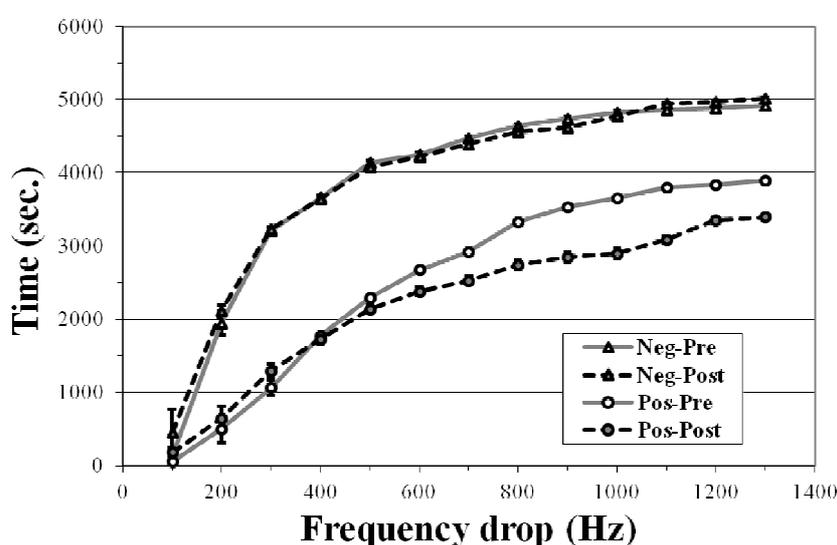


Figure 4. Time-frequency-drop plots for positive and negative samples before and after amplification

It should be noted that the per cent coefficient of variation (% CV) was quite high for the frequency change of less than 500 Hz as shown in Figure 4. However, it was less than 35 Hz for the frequency changes greater than 500 Hz in negative samples (Neg-Pre and Neg-Post) and positive samples before amplification (Pos-Pre), while it was in the range of 40-230 Hz for positive sample after amplification (Pos-Post). The high % CV or fluctuation in the Pos-Post samples could have resulted from the movement of the amplified DNA chains. Moreover, it is obvious that the overall frequency change rates of the negative samples, both Neg-Pre and Neg-Post, were lower than those of the positive ones (Pos-Pre, Pos-Post). The changes in frequency of the negative samples (Neg-Pre and Neg-Post) were very slow at the beginning (~13 sec/Hz) and higher after 1 hr (~1 sec/Hz). No differences were observed between the negative samples before and after amplification. However, the positive samples (Pos-Pre and Pos-Post) showed a rapid change from ~5 sec/Hz to ~1 sec/Hz from the beginning to 1 hr later respectively. This rapid change might have been due to the DNA template being added to the positive samples and also to the amplified DNA products by LAMP producing various long chains of DNA. According to Tsortos and co-workers [16], longer

chains of DNA induce higher viscosity, which leads to a faster drop in frequency. This can be observed in Figure 4, where the positive sample after amplification (Pos-Post) took about 3300 sec. for a frequency drop of 1300 Hz, while the positive sample before amplification (Pos-Pre) and the negative samples before (Neg-Pre) and after (Neg-Post) LAMP took 3800, 4900 and 4900 sec. respectively for this drop.

Classification using radar graphs

Although it is possible to distinguish the positive and negative samples groups (Figure 4), it is still difficult to classify individual samples due to variation. In our attempt to classify individual samples, mathematical models were applied to categorise the four groups of samples. Curve fitting using 4 polynomial functions, namely linear function ($y = a_0 + a_1t$), 2nd-order ($y = a_0 + a_1t + a_2t^2$), 3rd-order ($y = a_0 + a_1t + a_2t^2 + a_3t^3$) and 4th-order polynomial functions ($y = a_0 + a_1t + a_2t^2 + a_3t^3 + a_4t^4$) was used on frequency-time plots. Means of coefficients (a_i) and coefficient of determination (R^2) were used in the analysis. The ratio of the coefficient before amplification to that after amplification for each sample was calculated and shown in Table 1. All the variables are defined as follows.

$$a_i' = a_i(\text{Post}) / a_i(\text{Pre})$$

$$a_i' = \text{ratio of coefficient before and after amplification for each polynomial coefficient at order } i \text{ and } R^2$$

$$a_i(\text{Post}) = \text{means of coefficients in each polynomial order for sample after amplification}$$

$$a_i(\text{Pre}) = \text{means of coefficients in each polynomial order for sample before amplification}$$

$$i = \text{order of coefficient}$$

For a clearer representation, the data were plotted as radar charts (Figure 5). Positive and negative samples could not be separated using linear and 2nd-order polynomial functions. However, they could be clearly separated using 3rd- and 4th-order polynomial functions. Thus, it is possible to identify whether a sample has DNA template by comparing coefficient a_i' . Values of a_2' , a_3' and a_4' for the 3rd- and 4th-order polynomial functions respectively significantly increased in the positive sample. This indicates that as time passes, the change in frequency is dominated by higher order coefficients. Thus, a certain time period is necessary in determining the frequency-time plot to achieve successful classification. In this case 3000 seconds (~50 min.) was needed for the classification.

Principal component analysis (PCA)

PCA was also applied to classify the samples. However, when the same data set was used as in the previous analysis, the samples could not be visually classified. The data used in the feature extraction for PCA was therefore slightly modified. The data set used in PCA was collected when the frequency change was larger than 200 Hz after sample loading at the points 'b' and 'e' (Figure 3). The procedure for coefficient extraction was the same as that for radar graph analysis. The data from the coefficient extraction for each sample were plotted in the PCA feature space. Figure 6 shows PCA scores in 2-dimensional feature space of PC1 vs. PC2, PC1 vs. PC3 and PC2 vs. PC3. PCA scores plot of PC1 vs. PC2 shows quite a good separation between positive and negative samples, although one of the samples is the outlier (Figure 6A). The separation of positive samples before (Pos-Pre) and after (Pos-Post) amplification is clearly observed, while the separation of

negative samples before (Neg-Pre) and after (Neg-Post) amplification cannot be achieved. The PCA score plot of PC2 vs. PC3 in Figure 6C also shows that the group of positive samples after amplification (Pos-Post) can be isolated from the others, as was the case in the PC1 vs. PC2 plot. This confirms that the target DNA was successfully amplified in the positive samples during the LAMP process and no amplification occurred in the negative samples. However, it should be noted that it is difficult to group the samples using PCA score plot of PC1 vs. PC3. This implies that even though the score of PC2 is not the highest, it plays an important role in grouping the samples as shown in Figures 6A and 6C.

Table 1. Mathematical values obtained from all groups of samples

Function	Sample	R^2	a_0	a_1	a_2	a_3	a_4
Linear	Neg-Pre	0.60	-2228	-0.38			
	Neg-Post	0.65	-2297	-0.50			
	Neg-Post/Neg-Pre(a_i')	1.09	1.03	1.33			
	Pos-Pre	0.65	-2371	-0.39			
	Pos-Post	0.67	-2489	-0.53			
	Pos-Post/Pos-Pre (a_i')	1.03	1.05	1.34			
2nd-Order	Neg-Pre	0.79	-2491	0.41	-3.75E-04		
	Neg-Post	0.84	-2588	0.53	-6.63E-04		
	Neg-Post/Neg-Pre(a_i')	1.06	1.04	1.28	1.77		
	Pos-Pre	0.83	-2611	0.33	-4.00E-04		
	Pos-Post	0.83	-2735	0.40	-7.23E-04		
	Pos-Post/Pos-Pre (a_i')	1.01	1.05	1.23	1.81		
3rd-Order	Neg-Pre	0.87	-2288	-0.81	1.15E-03	-5.25E-07	
	Neg-Post	0.90	-2387	-0.87	1.54E-03	-1.13E-06	
	Neg-Post/Neg-Pre(a_i')	1.04	1.04	1.08	1.34	2.14	
	Pos-Pre	0.89	-2442	-0.65	8.75E-04	-5.00E-07	
	Pos-Post	0.89	-2570	-0.86	1.92E-03	-1.48E-06	
	Pos-Post/Pos-Pre (a_i')	1.00	1.05	1.32	2.20	2.95	
4th-Order	Neg-Pre	0.89	-2400	0.30	-1.35E-03	1.65E-06	-5.25E-10
	Neg-Post	0.92	-2494	0.35	-1.86E-03	2.75E-06	-1.38E-09
	Neg-Post/Neg-Pre(a_i')	1.03	1.04	1.16	1.38	1.67	2.62
	Pos-Pre	0.91	-2539	0.22	-9.50E-04	7.75E-07	-3.85E-10
	Pos-Post	0.90	-2661	0.17	-1.44E-03	2.65E-06	-2.05E-09
	Pos-Post/Pos-Pre (a_i')	1.00	1.05	0.80	1.51	3.42	5.31

Note: R^2 = coefficient of determination; a_0 = mean of polynomial coefficient at order 0; a_1 = mean of polynomial coefficient at order 1; a_2 = mean of polynomial coefficient at order 2; a_3 = mean of polynomial coefficient at order 3; a_4 = mean of polynomial coefficient at order 4; a_i' = ratio of coefficient before and after the amplification for each polynomial coefficient at order i and R^2 ; E = times ten raised to the power of the value of the exponent

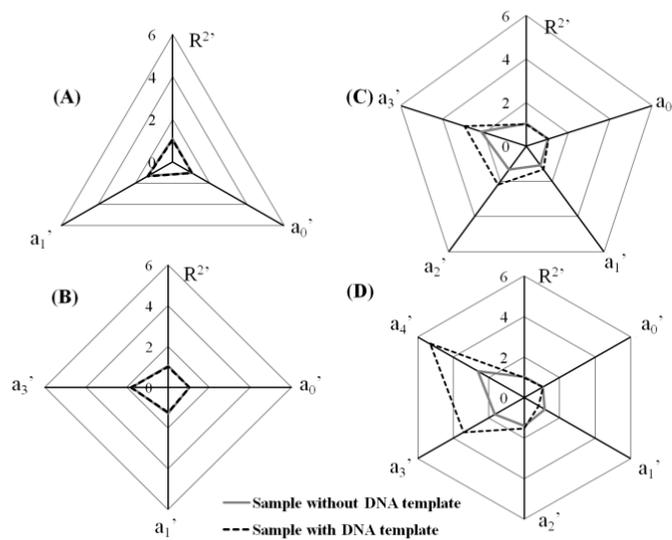


Figure 5. Rader charts of ratio of coefficients before and after amplification from positive and negative samples using four mathematical models: (A) linear; (B) 2nd-order polynomial; (C) 3rd-order polynomial; (D) 4th-order polynomial

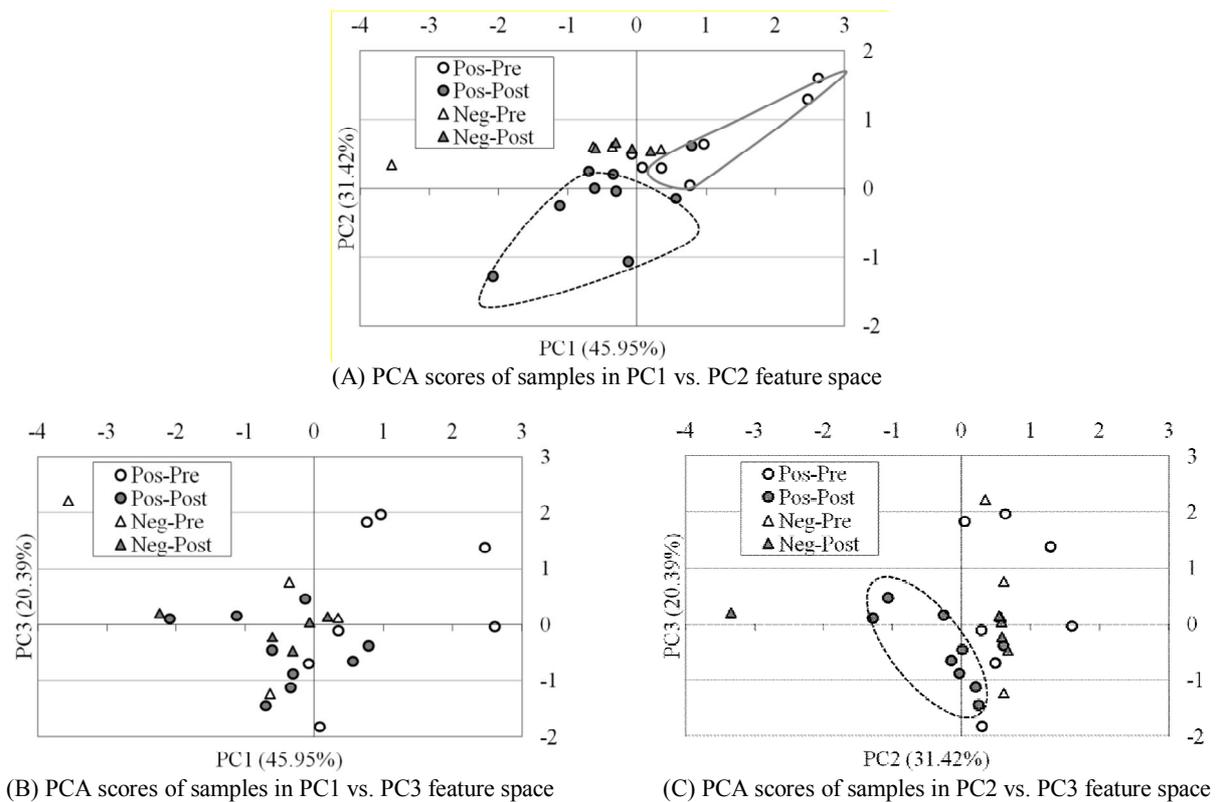


Figure 6. PCA of the four groups of samples: (A) PCA scores of samples in PC1 vs. PC2 feature space; (B) PCA scores of samples in PC1 vs. PC3 feature space; (C) PCA scores of samples in PC2 vs. PC3 feature space

CONCLUSIONS

This study shows the feasibility of separating positive and negative samples both before and after amplification by LAMP using QCM sensor. This label-free technique can amplify target DNA at a single temperature without the need of a thermocycler and demonstrates an effective method for qualitative DNA detection using a combination of LAMP and QCM, together with simple mathematical functions.

ACKNOWLEDGEMENTS

This research was financially supported by the Chulalongkorn University Centenary Academy Development Project. Dr. Pakpum Somboon is gratefully acknowledged for kindly commenting on the PCA. The University Development Commission at the Ministry of University Affairs is thanked for W.B.'s scholarship.

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